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Microfilament Contraction Promotes Rounding of Tunic Slices: An Integumentary Defense System in the Colonial Ascidian *Aplidium yamazii*

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Abstract. In *Aplidium yamazii*, when a slice of a live colony (approximately 0.5 mm thick) was incubated in seawater for 12 h, the slice became a round tunic fragment. This tunic rounding was inhibited by freezing of the slices, incubation with Ca^{2+}-Mg^{2+}-free seawater, or addition of cytochalasin B. Staining of microfilaments in the slices with phalloidin-FITC showed the existence of a cellular network in the tunic. Contraction of this cellular network probably promotes rounding of the tunic slice. In electron microscopic observations, a new tunic cuticle regenerated at the surface of the round tunic fragments; the tunic cuticle did not regenerate in newly sliced specimens nor in specimens in which rounding was experimentally inhibited.

Based on these results, an integumentary defense system is proposed in this species as follows. (1) When the colony is wounded externally, contraction of the cellular network promotes tunic contraction around the wound. (2) The wound is almost closed by tunic contraction. (3) Tunic contraction increases the density of the filamentous components of the tunic at the wound, and it may accelerate the regeneration of tunic cuticle there.

Introduction

The integumentary tissues of metazoans commonly function to protect the body from a hostile environment and as a transporting surface, and they display various structures. Some of these tissues have a keratinous, collagenous, or chitinous cuticle, and some have a ciliated surface or a mucous layer. The body of urochordates (tunicates) is usually covered with a leathery or gelatinous matrix called the tunic. The tunic is a peculiar integumentary tissue in metazoans for the following two reasons. First, the tunic contains cellulosic fibers that link proteins (De Leo *et al.*, 1977; Van Daele *et al.*, 1992). Second, live free cells, called tunic cells, are distributed within the tunic, which is thus a mesenchyme-like tissue. In ascidians, the tunic cells are involved in various biological functions, such as phagocytic activity (De Leo *et al.*, 1981; Hirose *et al.*, 1994a), conduction of impulses (Mackie and Singla, 1987), and bioluminescence (Aoki *et al.*, 1989). Because it is unique, investigations on tunic morphology and functions may lend perspective to our understanding of integumentary and mesenchymal tissues.

Aplidium yamazii is a colonial ascidian (Polyclinidae, Aplousobranchia) with a gelatinous, transparent tunic in which elongated forms of zooids are embedded separately from each other. The tunic is overlaid by a thin cuticle, and the cuticular surface has numerous minute protrusions, each about 60 nm in height (Hirose *et al.*, 1990). The tunic cuticle has a dense structure that is probably an effective barrier to the invasion of microorganisms into the tunic. Various kinds of tunic cells are distributed in the tunic of this species (Hirose *et al.*, 1994b), and no colonial vascular network (tunic vessels) connects the zooids. When tunic slices are incubated in seawater, they spontaneously round up to form tunic balls. This phenomenon is presumed to represent the mechanism by which external injuries of the tunic are healed. In this study, we examine the mechanism of rounding of the slices and discuss its functions as an integumentary defense system of this tissue.

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Materials and Methods

Animals

The colonies of *Aplidium yamazii* were collected in Nabeta Bay, Shimoda (Shizuoka Prefecture, Japan). They were temporarily kept in running seawater in the laboratory or reared in a culture box immersed in Nabeta Bay. *A. yamazii* forms a relatively flat colony, about 2 to 3 mm thick, spreading on a flat substratum; its rod-shaped zooids are embedded separately in a common tunic (Fig. 1).

Tunic rounding assay

Colonies were transversely sliced with a razor blade into pieces that were 0.5 mm thick or less (*e.g.*, 7 × 2 × 0.5 mm). The tunic slices (Hirose *et al.*, 1994a) were composed of tunic, tunic cells, and small fragments of zooids; many of the zooid fragments were washed out. The fresh tunic slices were placed in a plastic petri dish filled with filtered seawater (FSW) or artificial seawater (ASW), and were incubated overnight at 17° to 20°C. During the incubation, each specimen shrank and became a single tunic ball.

Video recording of tunic rounding

The tunic slices were put in a 100-ml beaker filled with FSW (16°–18°C), and the process of tunic rounding was recorded with a time-lapse videocassette recorder (AG-6010; National, Osaka, Japan) and a video camera (WV-1800; National) equipped with a 55-mm macro lens (Micro-Nikkor; Nikon, Tokyo). Recording was performed at about 1/60 of the actual speed. The time course of transformation was analyzed by hourly measurements of the length of the longest diagonal line that could be drawn within the profile of the rounding specimens.

Tunic rounding assay under experimental conditions

Some of the slices were frozen at −20°C, thawed at room temperature, and then incubated in ASW.

Live slices were incubated in three kinds of experimental media: Ca^{2+}-Mg^{2+}-free artificial seawater (CMF-ASW), various concentrations of colchicine-ASW, and various concentrations of cytochalasin B-ASW. Because cytochalasin B was dissolved in dimethylsulfoxide (DMSO) before dilution in ASW, the assay was also carried out in 1% DMSO-ASW as a control. All media also contained penicillin (100 IU/ml) and streptomycin (1 μg/ml).

Staining with phalloidin-fluorescein isothiocyanate (FITC)

Microfilaments were visualized in colony slices by labeling with phalloidin-FITC. The specimens were fixed with 3.5% formaldehyde in Ca^{2+}-free artificial seawater (CF-ASW) for 10 min, made permeable with 0.1% Triton X-100 in CF-ASW for 5 min, and washed with phosphate-buffered saline (PBS). They were incubated with 1 μg/ml phalloidin-FITC in PBS for 30 min and then were rinsed extensively with PBS. Some fixed specimens were embedded in O.C.T. compound so that cryostat sections could be made. Sections that were 20 μm thick were stained with 2 μg/ml phalloidin-FITC in PBS for 30 min and rinsed with PBS. These specimens were observed under a microscope equipped with epifluorescence and Nomarski differential interference contrast optics.

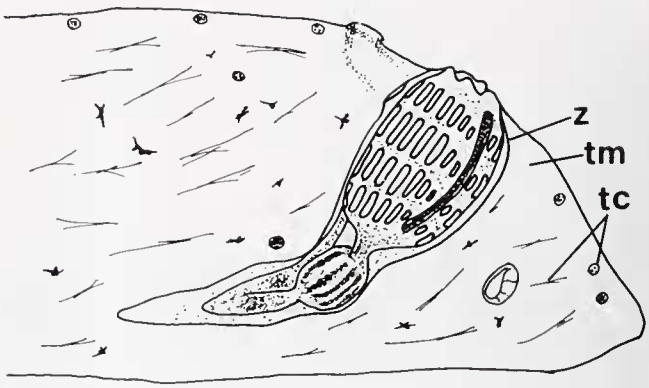

Figure 1. Schematic drawing showing the frontal section of a colony. A zooid (z) is embedded in the tunic matrix (tm). Tunic cells (tc) are distributed throughout the tunic. There are no blood vessels in the tunic.

Chemicals

ASW, CF-ASW, and CMF-ASW were obtained from Jamarine Lab., Osaka, Japan. Colchicine, cytochalasin B, and phalloidin-FITC were from Sigma Chemical Co., St. Louis, Missouri. O.C.T. compound was from Miles Inc., Naperville, Illinois.

Electron microscopy

The specimens were fixed in 2.5% glutaraldehyde-ASW or 2.5% glutaraldehyde-0.1 *M* cacodylate-0.45 *M* sucrose (pH 7.4). They were rinsed with the same buffer, postfixed in 1% osmium tetroxide-0.1 *M* cacodylate (pH 7.4), and dehydrated through graded ethanol. For scanning electron microscopy (SEM), the specimens were dried in a critical-point dryer, coated with Au-Pd, and examined in a Hitachi S-570 scanning electron microscope at 20 kV. For transmission electron microscopy (TEM), the dehydrated specimens were cleared with *n*-butyl glycidyl ether and embedded in low-viscosity epoxy resins. Thin sections were stained with uranyl acetate and lead citrate and were examined in a Hitachi HS-9 transmission electron microscope.

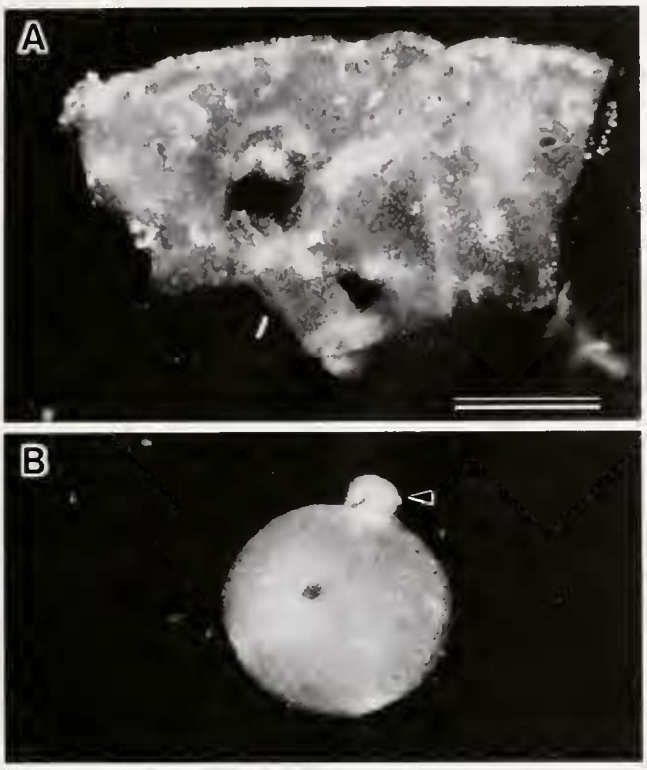

Figure 2. A freshly cut tunic slice (A) and a rounding tunic specimen after incubation in FSW for 24 h (B). Arrowhead indicates a zooid fragment extruded from the tunic ball. Magnifications of these two figure parts are the same. Scale bar = 1 mm.

Results

Freshly cut tunic slices were basically thin rectangular pieces of tunic (Fig. 2A), and each of them rounded up into an elastic tunic ball after incubation in ASW (Fig. 2B). The tunic ball was completely filled with tunic matrix, and no hollows remained. During the incubation, some of the zooid fragments were pushed out from the rounding tunic specimens, and the others were packed inside the tunic ball, but rounding occurred even if every zooid fragment in a tunic slice was lost. Tunic rounding, therefore, did not depend on the presence of zooids or zooid fragments. The size of a tunic ball depended on the initial size of the slice and the quantity of zooid fragments that were lost during rounding. For instance, tunic slices of about 2.5 × 5 × 0.5 mm transformed to tunic balls of 2 to 2.7 mm in diameter. In a few cases, one tunic slice would round up into two or three balls connected to each other by thin strands of tunic material, or a tunic slice deformed into a rodlike or irregularly shaped mass of tunic. Within a tunic ball or deformed tunic mass, the tunic cells were alive and some were motile. Noticeable tunic rounding began 4 to 5 h after a slice was prepared, and proceeded gradually for about 20 h; typical time courses are shown in Figure 3.

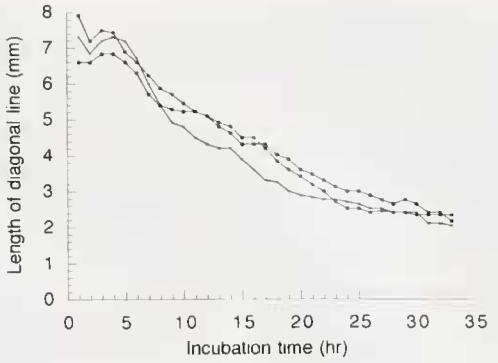

Figure 3. Time course of rounding of the tunic slices. The length of the longest diagonal line was measured in three specimens every hour during the incubation in FSW.

Table I shows the results of the tunic rounding assay under experimental conditions. In ASW (control), most of the tunic slices became tunic balls; those that did not form balls stuck on the surface of the petri dishes and rounding or deformation of the tunic partially occurred in the periphery or in some small areas in these slices. Tunic slices that were frozen and thawed did not round up, and the hardness and shapes of these tunic slices were almost unchanged (Fig. 4A). Tunic rounding was completely inhibited in CMF-ASW, and the tunic slices were transformed into disorganized soft gel (Fig. 4B). These specimens were so soft that they were easily taken to bits by handling with forceps. Cytochalasin B also inhibited the rounding, with 1 μg/ml of cytochalasin B being enough for complete inhibition. These tunic slices became softer than either new or frozen slices, and they were swollen to some extent (Fig. 4C). Because tunic rounding normally occurred in 1% DMSO-ASW, the small amount of DMSO

Table I

Rounding of colony slices under experimental conditions

Medium[a]	Concentration	No. of specimens	No. of rounding specimens (%)
ASW (control)		224	213 (95)
Freeze treatment		67	0 (0)
CMF-ASW		49	0 (0)
Cytochalasin B-ASW	5 μg/ml	95	0 (0)
	1 μg/ml	67	0 (0)
	0.5 μg/ml	18	13 (72)
	0.3 μg/ml	18	10 (56)
	0.1 μg/ml	37	32 (86)
DMSO-ASW	1%	56	51 (91)
Colchicine-ASW	10 μg/ml	30	28 (93)
	100 μg/ml	47	42 (89)

[a] ASW = artificial seawater; CMF-ASW = Ca^{2+}-Mg^{2+}-free artificial seawater; DMSO = dimethylsulfoxide.

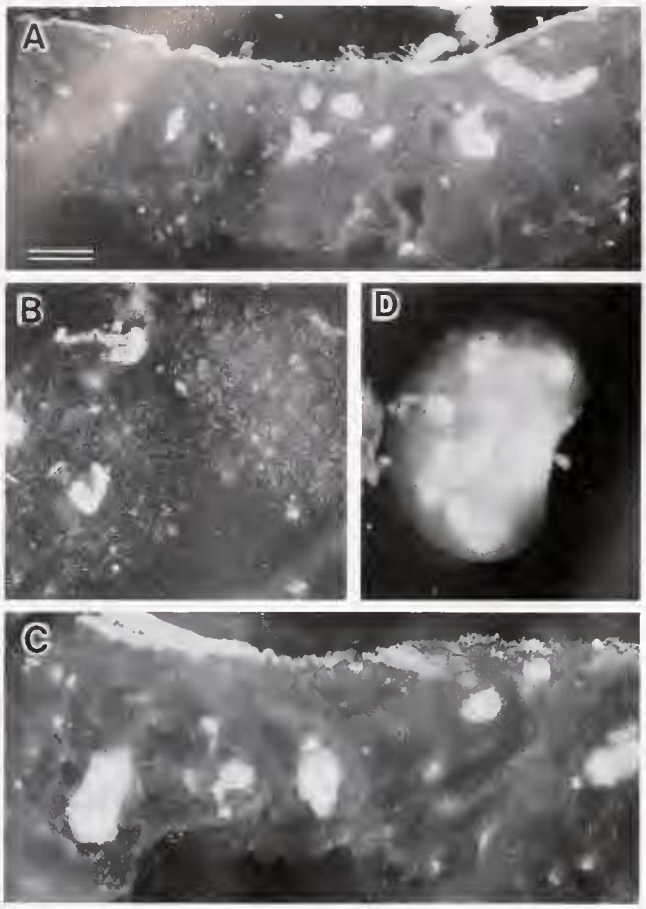

Figure 4. Tunic slices assayed under experimental conditions after a 24-h incubation: frozen and thawed once (A), incubated in Ca^{2+}-Mg^{2+}-free-ASW (B), incubated in cytochalasin B-ASW (5 μg/ml) (C), incubated in colchicine-ASW (10 μg/ml) (D). Tunic rounding occurred only in D. The slice was transformed into disorganized soft gel in B. Magnifications are the same for all parts of this figure. Scale bar = 1 mm.

contained in cytochalasin B-ASW did not have an inhibitory effect (see Table 1). Colchicine did not inhibit tunic rounding even in high concentrations (Fig. 4D).

The inhibitory effects on rounding by cytochalasin B suggest that microfilaments promote tunic rounding. Microfilaments in the tunic slices were visualized by labeling with phalloidin-FITC, which stained cellular microfilaments of tunic cells, particularly their filopodia. In *A. yamazii*, a type of tunic cell, called an elongated tunic cell, extended long cellular processes that were stained extensively with phalloidin-FITC. The elongated tunic cells appeared to form a cellular network by contacting each other with their cellular processes (Hirose *et al.*, 1994b) (Fig. 5A). But, this cellular network disappeared in tunic slices that had been incubated in CMF-ASW (Fig. 5B) or cytochalasin B-ASW (Fig. 5C). In these specimens, most of the tunic cells were almost spherical, with shortened filopodia or none at all. Cryostat sections were prepared from pieces of the colony (Fig. 5D) and rounding tunic balls (Fig. 5E). The cells were distributed rather uniformly in the tunics of colony pieces. After rounding, the number of tunic cells and filamentous materials increased significantly in cortical area of the specimens.

SEM observation of a fresh slice reveals the tunic matrix consisting of fine filamentous materials that densely intertwine; the surface of the slice has a sponge-like structure (Fig. 6A). After some rounding, a dense, sheetlike material covered the surface of the tunic ball, so the filamentous materials were not exposed (Fig. 6B); and TEM observation disclosed an electron-dense, thin layer covering the tunic matrix (Fig. 6C). This thin layer is a regenerating tunic cuticle. The ascidian tunic is always overlaid by a cuticle that entirely covers the matrix. In *A. yamazii*, the intact tunic cuticle has protrusions of about 60 nm (Hirose *et al.*, 1990) (Fig. 6D), and tiny protrusions were also found in the regenerating cuticle of the rounding tunic (Fig. 6C, arrows). Under experimental conditions in which the tunic did not round, the cuticle did not regenerate, and the filamentous materials remained exposed. In tunic slices that were frozen and thawed once, the surface structures were almost the same as those of freshly sliced specimens (Fig. 6E). In the tunic slices incubated in CMF-ASW or cytochalasin B-ASW (Fig. 6, F and G), the filamentous materials of the tunic were loosely packed in comparison with newly sliced specimens.

Discussion

Tunic slices of *Aplidium yamazii* gradually round up in seawater, usually becoming round tunic masses within 24 h. In other words, the superficial area of the specimen is minimized by rounding. Because tunic slices that had been frozen did not round, live tunic cells are probably necessary for rounding. Tunic rounding was also inhibited by cytochalasin B, which suggests that microfilaments are involved in the process. Phalloidin-FITC staining allowed visualization of the distribution of microfilaments in the tunic slices, and it revealed a network of tunic cells interconnected by their long filopodia. We deduce that tunic rounding is promoted by contraction of this cellular network in the tunic; that is, the network contracts, carrying with it the surrounding gelatinous tunic matrix. As shown in Figure 5E, the number of tunic cells and filamentous materials increase in the cortical area of the tunic ball. This suggests that shrinkage of the tunic occurs in the cortical area, and that the contraction of the cellular network probably promotes this tunic shrinkage. The complete inhibition of rounding in CMF-ASW may be caused by the disappearance of the cellular network (Fig. 5B). In contrast to microfilaments, microtubules are probably not essential for tunic rounding, because high concentrations of colchicine were not inhibitory.

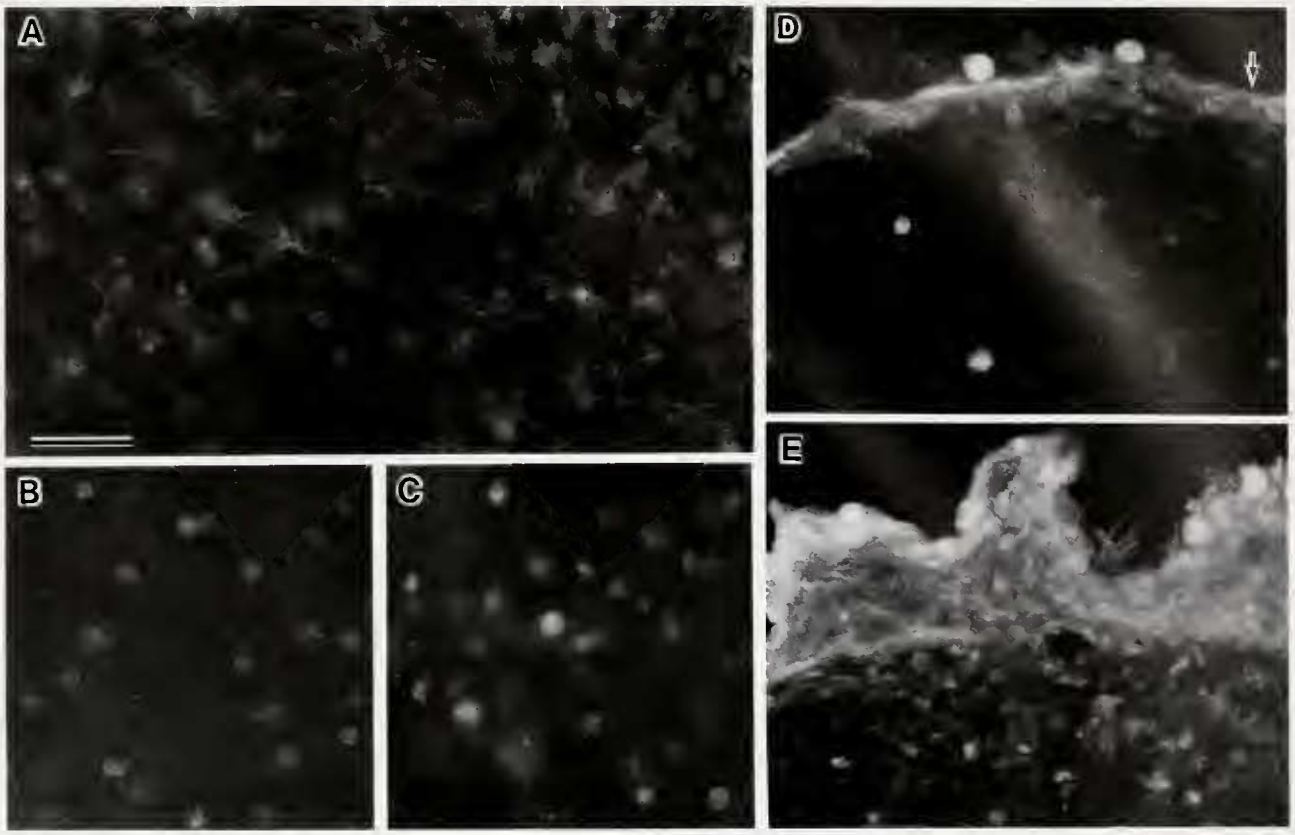

Figure 5. Microfilaments in the tunic specimens stained with phalloidin-FITC. In a newly sliced tunic, elongated tunic cells extending filopodia form a cellular network (A). The cellular network has disappeared in the tunic slices incubated in Ca^{2+}-Mg^{2+}-free-ASW (B), or incubated in cytochalasin B-ASW (10 μg/ml) (C). Cryostat sections of colony pieces (D) and rounding tunic balls (E), showing cortical area of the specimens. Arrow indicates tunic cuticle. Magnifications are the same for all parts of this figure. Scale bar = 50 μm.

The tunic cells forming the network have been described as "elongated tunic cells" (Hirose *et al.*, 1994b) and probably correspond to the "myocytes" described in *Diplosoma* species (Mackie and Singla, 1987). The myocytes also form a network in the tunic, and the net of myocytes itself is supposed to conduct impulses that trigger its contraction, according to electrophysiological studies (Mackie and Singla, 1987). Elongated forms of tunic cells were also reported in *Leptoclinides echinatus* (Hirose, 1992), although it is uncertain whether they form a network. On the other hand, similar types of tunic cells or tunic cell network have not been described in other colonial ascidians that have colonial vascular networks in the tunic, such as *Clavelina miniata* (Aoki *et al.*, 1989), *Perophora viridis* (Deck *et al.*, 1966), and *Botryllus* and *Botrylloides* species (Zaniolo, 1981; Hirose *et al.*, 1991). Although the epidermal cells of the vascular network show contractility (Mukai *et al.*, 1978) and impulse conductivity (Mackie and Singla, 1983), the tunic cell network may be uniquely developed in some colonial species that lack a colonial vascular network.

The fine-structure study revealed that the tunic cuticle, a thin, electron-dense layer, had regenerated in the rounding tunic ball and covered the entire surface. In contrast, the tunic cuticle was lacking and filamentous tunic materials were exposed at the surface of newly sliced tunic and in the specimens in which tunic rounding was inhibited. Rounding (or tunic shrinkage at the cortical area) may be necessary for cuticle regeneration at the exposed surface of the tunic.

Like newly sliced tunics, frozen specimens were gelatinous, and the filamentous tunic materials were intertwined densely at the surface. When the slices were incubated in CMF-ASW or cytochalasin B-SW, the specimens became much softer than newly sliced tunic or frozen specimens; moreover, the filamentous materials of the tunic were loosely packed, and the cellular network in the tunic was not present. The cellular network of elongated tunic cells may also be important for maintaining

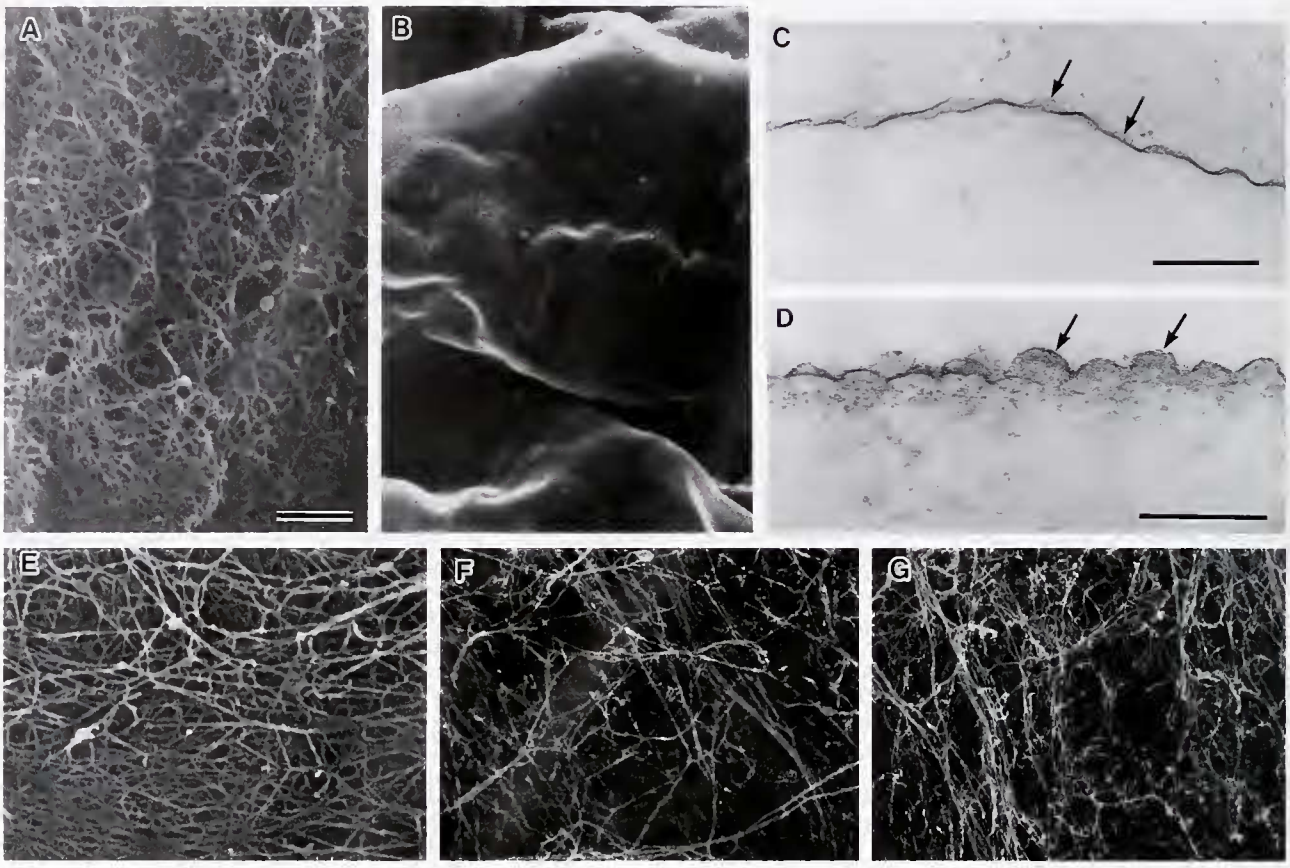

Figure 6. SEM (A, B, E, F, and G) and TEM (C and D) observation of tunic slices assayed under experimental conditions: newly sliced surface of the tunic (A), surface of rounding tunic (B and C), tunic cuticle of intact tunic (D), surface of tunic slice frozen and thawed once (E), tunic slice incubated in Ca^{2+}-Mg^{2+}-free-ASW (F), tunic slices incubated in cytochalasin B-ASW (10 μg/ml) (G). All SEM micrographs are at the same magnification. Arrows indicate some minute protrusions of the tunic cuticle. Scale bars = 1 μm in A, 0.2 μm in C and D.

tension in the tunic and the organization of the tunic filaments.

Tunic rounding is presumed to represent the healing mechanism that is initiated when the exterior of the tunic is injured. We propose the following integumentary defense system in the *A. yamazii* tunic. When the colony is externally wounded, contraction of the network of elongated tunic cells promotes contraction of the tunic around the wound. Tunic contraction almost closes the wound, and it minimizes the exposed area that lacks tunic cuticle. At the same time, tunic contraction increases the density of the filamentous components of the tunic at the wound, and it may accelerate the regeneration of tunic cuticle so as to cover the exposed surface of the wound. The tunic cuticle has a dense structure that is effective in preventing the invasion of microorganisms. Tunic rounding is, however, a slow process in which noticeable rounding begins 4–5 h after production of a slice, so this phenomenon may not be an effective defensive mechanism in the early stages after an injury. If, in *A. yamazii*, microorganisms invade through the wounded part before the completion of cuticle regeneration, phagocytic tunic cells might be expected to phagocytize those invaders (Hirose *et al.*, 1994a). This integumentary defense system is unique among metazoans, and it appears to be especially suited to the organization of this species, which has a large amount of tunic outside the epidermis and no vascular network in the tunic. In this system, the contractile cellular network in the tunic may work like the dermal or epidermal muscle does in other metazoans.

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